Report

Acute toxicity of ██████████ to fish

Laboratory Project Identification

Serial No. of experiment: ██████████

Serial No. of report: ██████████

Test facility

Institute of pesticide and environmental toxicology, Zhejiang University

(IPET-ZJU), Hangzhou 3100929, CHINA

Project completion date

23-Jun-2010

Catalog

1. Statement of GLP compliance

Study title: Acute toxicity of ████████ to fish

The study described in this report has been correctly reported and was conducted in compliance with *The Organization for Economic Cooperation and Development (OECD) Series on Principles of Good Laboratory Practice and Compliance Monitoring*[1]. This report recorded, accurately and in detail, method, step, and result of the experiment. The result reflected, accurately and fully, original data of the experiment.

The testing result is responsible for the submitted material only.

Manager: ██████████████████

Signa████ ████ ─Date─ *June 23, 2010*

Study director ████████████████

Signatur█ ██████████ Date *2010. 6. 23*

2. Quality assurance statement

Study title: Acute toxicity of ███████ to fish

This report was inspected by the IPET-ZJU Quality Assurance Unit to confirm that the methods and results accurately and completely reflect the raw data.

The dates of Quality Assurance inspections are given below. April

Type of inspections	Phase / Section	Inspection date		Reporting date
		Start	End	
Study	Protocol	28-Apr-2010	28-Apr-2010	28-Apr-2010
Process	Test substance handling, exposure, and observation/measuring	05-May-2010	20-May-2010	20-May-2010
Study	Report	20-Jun-2010	20-Jun-2010	20-Jun-2010

Head of quality assurance: ███████████

Signat ███████████   Date 2010.6.23

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3. Abstract

Acute toxicity test of ▮▮▮▮ was tested with zebrafish *Brachydanio rerio*. The study procedure described in this report was based on the OECD guideline No. 203, 1992 [2].

The batch of ▮▮▮▮ tested was a dark purple powder with a purity of >99% and not completely soluble in test medium at the concentrations tested.

The test was performed by exposing 10 fish to a filtered solution prepared at a loading rate of 100 mg/L and a blank-control. The test solution was prepared by magnetically stirring of the dispersion for 8 hours, after which it was filtered over a paper filter. The total test period was 96 hours. Samples for analysis were taken at the start, after 24, 48, 72, and 96 hours after the exposure.

Chemical analysis showed that the measured initial concentration was 44.15 mg/L. After 24 hours of exposure, the measured concentration was 43.53 mg/L. At the end of the 96h exposure period the measured concentration decreased to 41.60 mg/L.

▮▮▮▮ induced no visible effects in zebrafish exposed to a filtrate prepared at a loading rate of 100 mg/L. This suggested that LC_{50} of the test substance were above this concentration level. Due to its soluble limit, the average exposure concentration of ▮▮▮▮ was measure to be 42.88 mg/L.

4. Introduction

4.1. Sponsor

Name   ▬▬▬▬▬▬

Address   ▬▬▬▬▬▬▬

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4.2. Test facility

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Manager   ▬▬▬▬▬▬▬

Study director   ▬▬▬▬▬

Principal   ▬▬▬

investigator   ▬▬▬▬

▬▬▬

Study plan   Start   12-Jun-2010

Completion   16-Jun-2010

4.3. Aim of the study

The purpose of the study was to evaluate the test substance for its ability to generate acute toxic effects in *Brachydanio rerio* during an exposure period of 96 hours and, if possible, to determine the LC_{50} at all observation times.

4.4. Guidelines

The study procedure described in this report was based on the OECD guidelines for Testing of Chemicals, guideline No. 203: "Fish Acute Toxicity Test", adopted 17 July, 1992 [2].

4.5. Storage and retention of records and materials

Records and materials pertaining to the study including protocol, raw data, test substance (except those requiring refrigeration or freezing) and the final report are retained in IPET-ZJU archives for a period of at least 10 years after finalization of the report. After this period, the sponsor will be contacted to determine whether the raw data and the test material should be returned to them, retained or destroyed on their behalf.

Test substance requiring refrigeration or freezing will be retained by IPET-ZJU for as long as the quality of the test material permits evaluation but no longer than three months after finalization of the report.

4.6. Definitions

Fish were considered **dead** when no reaction was observed after touching the caudal peduncle and visible breathing movements were absent. In addition, fish that were convulsing or showing other severe forms of distress not considered transient in nature and likely to become more severe before the exposure is terminated, will be sacrificed for humane reasons. These fish will be treated as having died in the test.

LC_{50} is the concentration killing 50% of the fish after a defined period of exposure.

Diluent refers to solutions of the test substance.

Dilution is the medium used for preparing test solutions.

5. Text

5.1. Materials and methods

5.1.1. Chemical (Test substance)

Name ███

Batch No. ███

Chemical   name
(IUPAC) ███

Molecular formu███

Molecular
structure ███

Molecular
structure   1317.5

Purity   >99%

Melting/degrading
point   To degrade at 250℃

Appearance   Dark purple powder

Log P_{ow} ≥3.3

5.1.2. Fish

Zebrafish, *Brachydanio rerio*, were obtained from Hangzhou Yangfan Tropic Fish Hatchery. Size of the fish was in average length of 3.04 cm and average weight of 0.180 g. The fish were domesticated in dechlorinated rust-free tap water of 23-25℃. The total hardness of the water, as counted by $CaCO_3$, was 60~80 mg/L as measured by the method described in DZ/T 0064.15-93[3]. Light cycle was adjusted to 16 h light vs 8 h dark, and light intensity was kept in rang of 600~800Lux. During the domestication, the fish were fed at least once daily until 24 hours before test. Total mortality of the population was record to be 5% during seven day acclimatization period before the test.

5.1.3. Diluent (for limit test)

To prepare the diluent, a weighed amount of 1000 mg test substance was added to each 10 L dilution. After a stirring of 8 hours, the mixture was filtrated with qualitative filter paper (ø15 cm, fast speed) to remove undissolved test substance particles.

5.1.4. Test procedure and conditions

Test duration	96 hours
Exposure Strategy	Static
Test vessels	Glassware with diameter of 30 cm and height of 30 cm.
Source of dilution	The same as that used for fish domestication.
No. of fish per group	10 fish per group, to be introduced into the diluent within 30 minutes after preparation.
Light cycle	12h light *vs* 12h dark
Illumination	Fluorescent lamp, with light intensity of 600~800Lux
Aeration	The diluent was not aerated during the test.
Feeding	The fish was not fed during the total test period.
Euthanasia	Dying fish were rapidly killed by exposing them to aqueous

solution of clove oil of 250mg/L.

5.1.5. Measurements and recordings

Poisoning symptoms	The poisoning symptoms were examined on a time schedule of 0~8h, 8~24h and 24~96h.
Mortality	The mortality was recorded at 24th h, 48th h, 72nd h, and 96th h after the exposure. The dead fish were cleaned out in time.
Water quality parameters	The temperature, pH value, and dissolved oxygen of diluent was determined at 0th h, 24th h, 48th h, 72nd h, and 96th h according to GB/T 13195-1991[4], GB/T 6920-1986[5], and GB/T 11913-1989[6], respectively.

5.1.6. Sampling for analysis of test concentrations

Samples were taken at 0th h, 24th h, 48th h, 72nd h, and 96th h from the test concentration and the blank-control. Sample of about 50 mL was taken each time from each of the two vessels. The method for sample analysis is described in Appendix 1.

5.1.7. Data handling

The average exposure concentration was calculated as:

$$\frac{24 * \sqrt{C_{t=0} \times C_{t=24}} + 72 * \sqrt{C_{t=24} * C_{t=96}}}{96}$$

Where, $(C_{t=0})$, $(C_{t=24})$, and $(C_{t=96})$ is the concentration of the test substance measured in the samples taken at the start, after 24th h, and 96th h of exposure, respectively.

5.1.8. Acceptability of the test

- No abnormal behavior (including mortality) was observed in the control

group.

- The temperature was maintained within 23-25°C throughout the test.

- The oxygen concentration was maintained at least 60% of the air saturation value (i.e. >5.05mg/L at 24℃) throughout the test.

- The actual test concentration maintained higher than 80% of the initial value throughout the test.

5.1.9. List of protocol deviations

There were no deviations from the protocol.

5.2. Results

5.2.1. Poisoning symptoms

$0\sim8^{th}h$ No observable symptoms were observed in both control and treatment group.

$8\sim24^{th}h$ No observable symptoms were observed in both control and treatment group.

$24\sim96^{th}h$ No observable symptoms were observed in both control and treatment group.

5.2.1 Mortality

No fish was found die during the period of 96 hours (Table 1).

Table 1. Mortality of zebrafish in limit test

Treatment	Number of fish	Cumulative number of dead				Total mortality (%)
		24th h	48th h	72nd h	96th h	
Blank control	10	0	0	0	0	0
Filtrate of 100 mg/L ███	10	0	0	0	0	0

5.2.2. Determination of effect concentrations

Chemical analysis indicated that the concentration of ███ measured in the samples taken at the start, after 24 h, and 96 h of exposure was 44.15 mg/L, 43.53 mg/L, and 41.60 mg/L, respectively (see Appendix 1). Average exposure concentration was, therefore, calculated to be 42.88 mg/L. Table 2 shows the effect of ███ based on the average exposure concentration to the fish.

Table 2. Effect of ███ to zebrafish in limit test

Parameter	Concentration (mg/L)	95% confidence interval
Observed NOEC	42.88[a]	-
24th h LC_{50}	>42.88[a]	-
48th h LC_{50}	>42.88[a]	-
72nd h LC_{50}	>42.88[a]	-
96th h LC_{50}	>42.88[a]	-

[a] Average concentration in a filtrate prepared at a loading rate of 100 mg/L.

5.2.3. Experimental conditions

The results of measurement of pH and dissolved oxygen concentrations are listed in Table 3 and Table 4, respectively, and the result of measurement of temperatures is shown in Table 5.

Table 3. Values of pH of the diluent and the control in limit test

Treatment	Day 0	Day 1	Day 2	Day 3	Day 4
Blank control	6.12	6.29	6.31	6.34	6.36
Filtrate of 100 mg/L ■■■■■	5.76	6.34	6.42	6.42	6.42

Table 4. Dissolved oxygen concentrations (mg/L) of the diluent and the control measured during the test

Treatment	Day 0	Day 1	Day 2	Day 3	Day 4
Blank control	7.25	7.06	7.18	7.12	6.80
Filtrate of 100 mg/L ■■■■■	6.92	7.08	6.59	6.70	6.44

Table 5. Temperatures (℃) of the diluent and the control in limit test

Treatment	Day 0	Day 1	Day 2	Day 3	Day 4
Blank control	23.8	24.1	24.4	23.2	24.4
Filtrate of 100 mg/L ■■■■■	23.4	24.1	24.6	23.3	24.4

5.3. Discussion and conclusion

Limit test indicated that ■■■■■ induced no visible effects in zebrafish exposed to a filtrate prepared at loading rate of 100 mg/L.

Chemical analysis showed that the concentrations of ■■■■■ changed from 44.15 to 41.60 mg/L during whole period of exposure (see Appendix 1), which were much lower than the loading rate of 100 mg/L. This might result from the low solubility of the test substance in dilution.

The temperatures, the dissolved oxygen concentrations, and the concentrations of the test substance fluctuated in range of 23.2 to 24.6℃, 6.44 to 7.25 mg/L, and 44.15 to 41.60 mg/L, respectively, throughout the period of 96 hour exposure. These fluctuations were in range of the acceptable limit, i.e. the fluctuation of temperatures were in range of 23.0~25.0℃, the concentrations of dissolved oxygen maintained higher than 60% of the air saturation value (i.e. >5.05 mg/L at 24℃), and the actual test concentration maintained higher than 80% of

the initial value (i.e. >35.32 mg/L).

Due to the low water solubility, it was reasonable to infer that in natural conditions the test substance would not reach concentration levels toxic to fish.

Binomial theory dictates that when 10 fish are used with zero mortality at loading rate of 100 mg/L, there is a 99.9 % confidence that the LC_{50} is greater than 100 mg/L [2].

Chinese EPA Guidelines for the Hazard Evaluation of New Chemical Substances (HJ/T 154-2004) gives a scheme for classification of acute toxicity of New Chemical to fish[7]:

LC_{50}＜1.00 mg/L	extremely toxic
1.00 mg/L≤LC_{50}＜10.0 mg/L	highly toxic
10.0 mg/L≤LC_{50}＜100 mg/L	moderately toxic
LC_{50}≥100mg *a.i.*/L	low toxic

According to above standard counted by the loading rate, ▮▮▮▮ can be considered as low toxic to zebrafish.

5.4. References

[1]. 经济合作和发展组织（Organization of Economic Cooperation and Development, OECD）——良好实验室规范——准则与管理系列。化学工业出版社，2006年6月

[2]. OECD guideline for testing of chemicals, No. 203. Fish, acute toxicity test. Adopted in July 17, 1992

[3]. 中华人民共和国地址矿产行业标准——地下水质检验方法——乙二胺四乙酸二钠滴定法测定硬度（DZ/T 0064.15-93）。中华人民共和国地址矿产部，1993－02－27批准，1993－10－01实施

[4]. 中华人民共和国国家标准——水质——水温的测定——温度计或颠倒温度计测定法（GB/T 13195-1991）。国家环境保护总局，1991－08－31批准，1992－06

－01实施

[5]. 中华人民共和国国家标准——水质——pH值的测定——玻璃电极法（GB/T 6920-1986）。国家环境保护总局，1986－10－10发布，1987－03－01实施

[6]. 中华人民共和国国家标准——水质——溶解氧的测定——电化学探头法（GB/T 11913-1989）国家环境保护局，1989－12－25批准，1990－07－01实施

[7]. 中华人民共和国环境保护行业标准——新化学物质危害评估导则（HJ/T 154—2004）。国家环境保护总局，2004－04－13发布，2004－06－01实施

Appendix 1. Chemical analysis

Determination of ███████ content in water samples

Laboratory Project Identification

Serial No. of experiment: ███████

Serial No. of report: ███████

Test facility

Institute of pesticide and environmental toxicology, Zhejiang University

(IPET-ZJU), Hangzhou 3100929, CHINA

Project completion date

19-Jun-2010

1. Report approval

Principal investigator: ████████████████

Signature: ███████   Date ‎ʃun 19, 2010

2. Introduction

2.1. Aims of the study

The purpose of the analytical study was to determine the actual concentrations of ████████ in samples taken from the test solutions used during an ecotoxicity test.

2.2. Study plan

Start   17-Jun-2010

Completion   17-Jun-2010

3. Materials and methods

3.1. Reagents

Methanol, Mili-Q water

3.2. Sample treatment

The sample was filtrated through a filter membrane of 0.45μm before being applied for ████████

3.3. Analysis

3.3.1. Equipment

██

████████████

3.3.2. Analytical conditions

Column   ████████████ █ ████████████

Mobile phase   methanol/Mili-Q water=70/30 (v/v)

Flow rate   1ml/min

Wavelength   ████████

Injection volume   20μL

3.3.3. Calibration solutions

The test substance was dissolved with methanol to obtain stock solution of 1000mg/L. Calibration solutions were prepared by further diluting the stock solution into 1.0, 5.0, 10.0, 20.0, and 100.0 mg/L, respectively.

3.3.4. Calibration curve

Signal responses of the equipment towards the calibration solutions were measured according to the ████████████ described in Section "3.3.2.". The linear calibration curve was constructed with DPS° by plotting signal responses of the equipment against concentrations of the calibration solutions:

$$R = a + b \times C$$

Where,   R   response to calibration solution

C   concentration of test substance in calibration solution (mg/L)

a   slope

b   intercept

3.3.5. Sample determination

$$C = (R - b) \times d \div a$$

Where,   C   concentration of the test substance in sample (mg/L)

R   response to sample solution

d   dilution factor

a   slope

b   intercept

4. Results

4.1. Calibration curve

Figure 1 shows diagram of the calibration curve. Figure 2 is shows chromatogram of a calibration solution.

4.2. Limit of detection

The detection limit was 0.1mg/L.

4.3. Samples

The results for measurement of the test substance in samples are summarized in Table 1. Figure 3 and Figure 4 shows chromatogram of a blank control and a filtrate of the test substance of 100 mg/L.
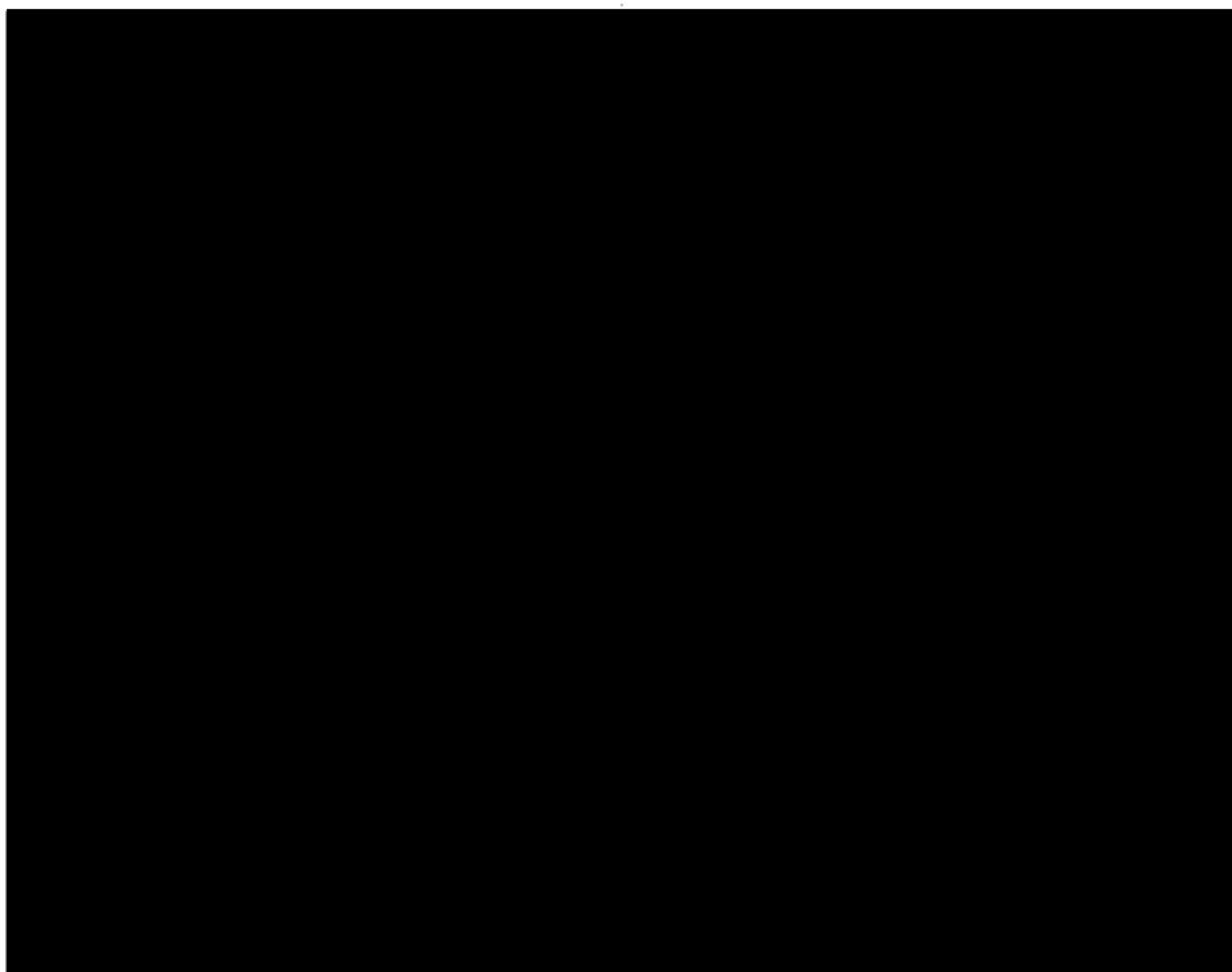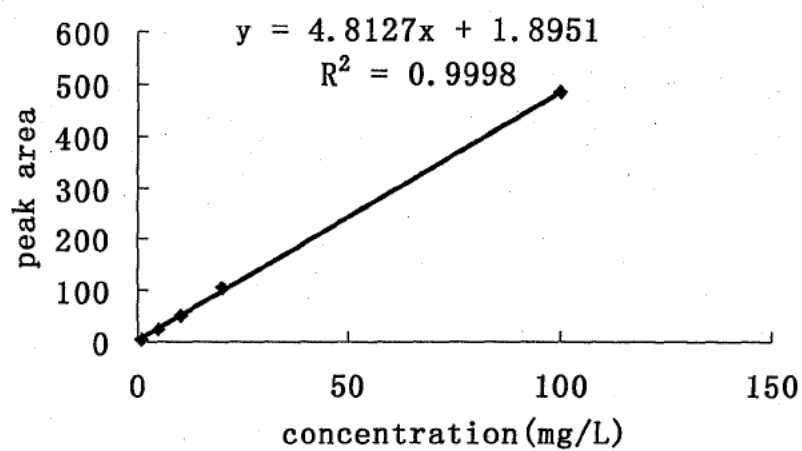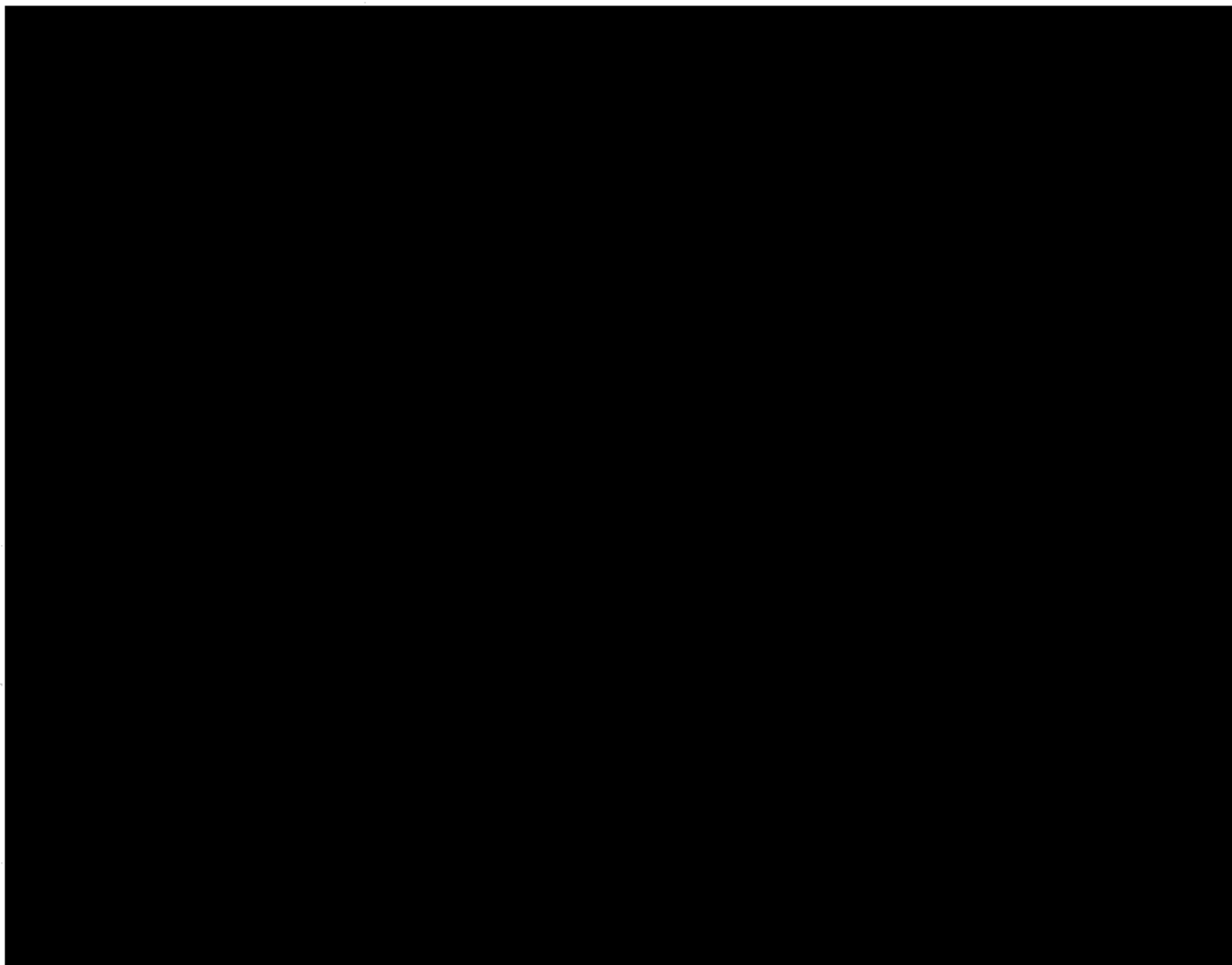
Figure 1. Linear equation for quantifying ███████

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Appendix 2. Reference test

24-hour toxicity of potassium dichromate to fish

The study procedure described in this report was based on the OECD guideline No. 203, 1992.

Start: 15-06-2010

End: 16-06-2010

This reference test was carried out to check the sensitivity of the test system as used by IPET-ZJU. The reference substance was potassium dichromate ($K_2Cr_2O_7$, 上海青析化工科技公司, Cas. 7778-50-9, Batch 090715).

Concentrstions: 99.0, 223, 149, 334, 501 mg/L in ISO-medium

Control: ISO-medium without test substance

Table 1. Incidence of mortality observed in the reference study

Concentrstions (mg/L)	Number of fish	Cumulative number of dead fish recorded at various time points after start of exposure				Total mortality
		0th h	8th h	16th h	24th h	
CK	10	0	0	0	0	0
99.0	10	0	0	0	0	0
149	10	0	0	0	0	0
223	10	0	0	0	0	0
334	10	0	0	3	5	80
501	10	0	2	4	4	100

During the test the pH, oxygen concentration and the temperature of the medium were within the optimal ranges for fish.

Under the conditions of the present test potassium dichromate induced no

lethal effects in fish at or below 223 mg/L. The 24h-LC50 was 316 mg/L. The range of the 24h-LC50 for fish is generally between 200 and 400 mg/L based on historical data of reference tests. The response observed in fish originating from the present batch falls within this range.

The raw data and report from this study are kept in the IPET-ZJU archives. The test described above was performed under GLP-conditions.